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Identification of a Kinase in Wheat Germ that Phosphorylates the Large Subunit of Initiation Factor 4F[1]

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JEAN HUMPHREYS, KAREN S. BROWNING, AND JOANNE M. RAVEL*
Department of Chemistry and Clayton Foundation Biochemical Institute, The University of Texas at Austin, Austin, Texas 78712-1096

ABSTRACT

A kinase has been isolated from wheat (*Triticum aestivum*) germ that phosphorylates the 220 kilodaltons (kD) subunit of wheat germ initiation factor (eIF) 4F, the 80 kD subunit of eIF-4B (an isozyme form of eIF-4F) and eIF-4G (the functional equivalent to mammalian eIF-4B). The kinase elutes from Sephacryl S-200 slightly in front of ovalbumin. The kinase phosphorylates casein and histone IIA to a small extent, but does not phosphorylate phosvitin. Of the wheat germ initiation factors, elongation factors, and small and large ribosomal subunits, only eIF-4F, eIF-4B, and eIF-4G are phosphorylated to a significant extent. The kinase phosphorylates eIF-4F to the extent of two phosphates per mole of the 220 kD subunit and phosphorylates eIF-4B to the extent of one phosphate per mole of the 80 kD subunit. The 26 kD subunit of eIF-4F and the 28 kD subunit of eIF-4B are not phosphorylated by the kinase. The kinase phosphorylates the 59 kD component of eIF-4G to the extent of 0.25 phosphate per mole of eIF-4G. Phosphorylation of eIF-4F and eIF-4B does not affect their ability to support the binding of mRNA to small ribosomal subunits *in vitro*.

Studies in mammalian cells have indicated that one of the ways by which the cells regulate the overall rate of protein synthesis is by phosphorylation-dephosphorylation of the protein synthesis initiation factors (11, 16, 17, 21). Several groups of investigators have shown that a correlation exists between phosphorylation of the α subunit of initiation factor 2 (eIF-2[2]) and inhibition of protein synthesis in mammalian cells (16, 17, 19, 21). Two ATP-dependent protein kinases, the hemin controlled repressor and the double stranded RNA-activated inhibitor, have been isolated from rabbit reticulocytes and shown to catalyze the phosphorylation of the α-subunit of eIF-2 (16, 17). Phosphorylation of the α-subunit of eIF-2 inhibits the recycling of eIF-2 (19, 21). eIF-2 kinases have also been found in other mammalian cells (11). The β-subunit of eIF-2 is phosphorylated by casein kinase II; however, phosphorylation of this subunit does not appear to affect the activity of the eIF-2 (16). Recent studies have shown that eIF-4B (9), eIF-4E (20), the 26 to 28 kD subunit of eIF-4F (10), as well as the ribosomal protein S6 (23) are phosphorylated *in vivo* in mammalian cells. In addition it has been shown that the degree of phosphorylation of these proteins varies during the cell cycle (4) and in response to hormones (11, 23), heat shock (10) or depletion of nutrients (9). The kinases responsible for the phosphorylation of mammalian eIF-4B, eIF-4E, and eIF-4F have not been identified, nor has it been shown that phosphorylation of these factors affects their activity. Recently Palen and Traugh (18) showed that phosphorylation of S6 by the mitogen-stimulated S6 kinase enhances the translation of globin mRNA in a reconstituted *in vitro* system.

We previously reported a kinase from wheat germ that phosphorylates the β-subunit of eIF-2, the 106 kD subunit of eIF-3 and three 60S ribosomal proteins from wheat germ (6). However, no change in the activity of these factors or the ribosomes could be demonstrated *in vitro* following phosphorylation by this kinase. We report here another kinase from wheat germ that phosphorylates the 220 kD subunit of wheat germ eIF-4F, the 80 kD subunit of eIF-4B,[3] and the 59 kD component of eIF-4G.[4]

MATERIALS AND METHODS

Materials. The 0 to 40% and 40 to 70% ammonium sulfate fractions, 80S ribosomes, 40S and 60S ribosomal subunits, and highly purified preparations of eIF-2, eIF-3, eIF-4A, eIF-1α, and EF-2 were obtained from extracts of wheat (*Triticum aestivum*) germ as described previously (14). eIF-4B (15), eIF-4F (15), and eIF-4G (7) were obtained by modifications of previously described procedures. Casein was purchased from Chemalog; phosvitin and histone IIA were kindly supplied by Dr. Gisela Kramer. [γ-^{32}P]ATP was obtained from New England Nuclear.

Purification of Kinase. The 0 to 40% ammonium sulfate fraction was chromatographed on DEAE-cellulose as previously described (14) except that the column was washed with 400 mL rather than 200 mL of buffer B (20 mM) Hepes/KOH, pH 7.6, 1 mM DTT, 0.1 mM EDTA, 10% glycerol) containing 150 mM KCl. Aliquots of the 150 mM KCl fractions were assayed for kinase activity as described below. The 150 mM KCl fractions containing kinase activity that were obtained from two DEAE-cellulose columns were pooled (generally 30 fractions), and the proteins were precipitated by the addition of solid ammonium sulfate to 70% of saturation. The precipitated proteins were suspended in a small amount (≈10 mL) of buffer B containing 100 mM KCl (B-100) and dialyzed against buffer B-100. The proteins were fractionally precipitated by the addition of a saturated solution of ammonium sulfate to yield 0 to 30%, 30 to 50%, and 50 to 70% fractions. The precipitated proteins were suspended in small amounts of buffer B-100 (≈1 ml) and dialyzed against buffer B-100. The majority of the kinase activity was

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[2] Abbreviation: eIF, eukaryotic initiation factor.

[3] This factor, designated eIF-4B by Lax *et al.* (14) and CSF by Seal *et al.* (22) contains two subunits (M_r = 80,000 and 28,000) and appears to be an isozyme of eIF-4F (14).

[4] This factor, designated eIF-4G by Browning *et al.* (7) and eIF-4B by Seal *et al.* (22), is a single polypeptide (M_r = 59,000) which appears to be the functional equivalent of mammalian eIF-4B.

recovered in the 30 to 50% fraction. After dialysis against buffer B-100 containing 50% glycerol, the sample (approximately 1.5 mL containing 32 mg of protein) was applied to a 145 mL Sephacryl S-200 column (1.5 × 85 cm) equilibrated in buffer B-100. The column was washed with buffer B-100 and fractions of 1.5 mL were collected. The fractions containing kinase activity (generally 3 fractions) were pooled, concentrated to about 1.5 mL in an Amicon concentrator fitted with a YM-10 membrane and dialyzed against buffer B-100 containing 50% glycerol. The sample (approximately 0.5 mL containing about 5 mg of protein) was chromatographed a second time on a 35 mL Sephacryl S-200 column (0.9 × 52 cm) equilibrated in buffer B-100. Fractions of 0.5 mL were collected.

Kinase Assay. The reaction mixture contained in 25 μL: 24 mM Hepes-KOH, pH 7.6, 2.4 mM $Mg(OAc)_2$, 100 mM KCl, 25 μM [γ-^{32}P]ATP (400–800 cpm/pmol), 10 μg of BSA, 20 to 25 pmol of eIF-4F, and kinase as indicated. After incubation at 25°C for 10 min, the reaction mixture was diluted with approximately 1 mL of HMK buffer (20 mM Hepes/KOH, pH 7.6, 2.5 mM $MgCl_2$, and 100 mM KCl) and was passed through a nitrocellulose filter (MFS, 0.45 μm) that had been soaked in 1 mM KPO_4, pH 6.8. The filter was washed with two 2 mL portions of cold HMK buffer, dried, and the amount of ^{32}P retained on the filter was measured in a scintillation counter. The values were corrected for the amount of ^{32}P retained on the filter (approximately 1 pmol) when eIF-4F and kinase were not added to the reaction mixture. A unit of kinase activity is defined as that amount which catalyzes the incorporation of 1 nmol of phosphate into eIF-4F under the conditions described above. Protein was measured by the method of Bradford (5) with BSA as the standard.

Table I. *Purification of 4F Kinase*

Step	Protein	Units	Specific Activity
	mg		*units/mg*
1. DEAE-cellulose[a]	97	4480	46
2. 30–50% $(NH_4)_2SO_4$	33	2208	67
3. Sephacryl S-200 I	3.6	767	213
4. Sephacryl S-200 II	1.0	349	349

[a] Derived from 400 g of wheat germ.

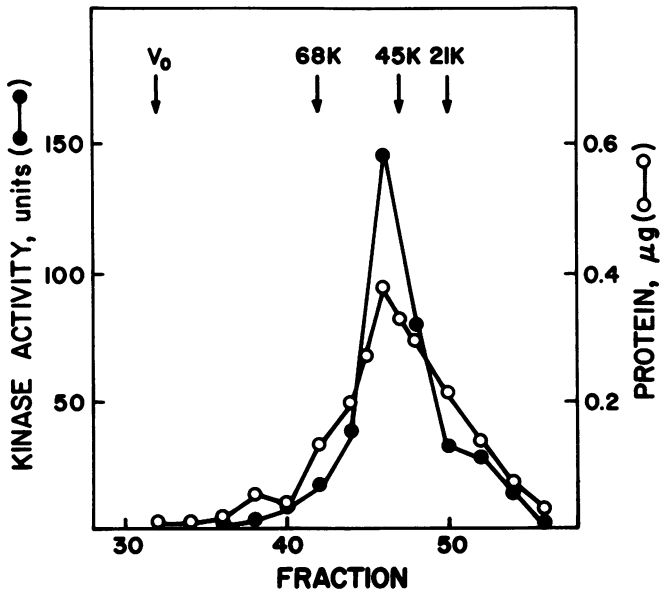

FIG. 1. Chromatogrpahy of the Sephacryl S-200 I preparation on a second Sephacryl S-200 column. Chromatography was carried out as described in "Materials and Methods."

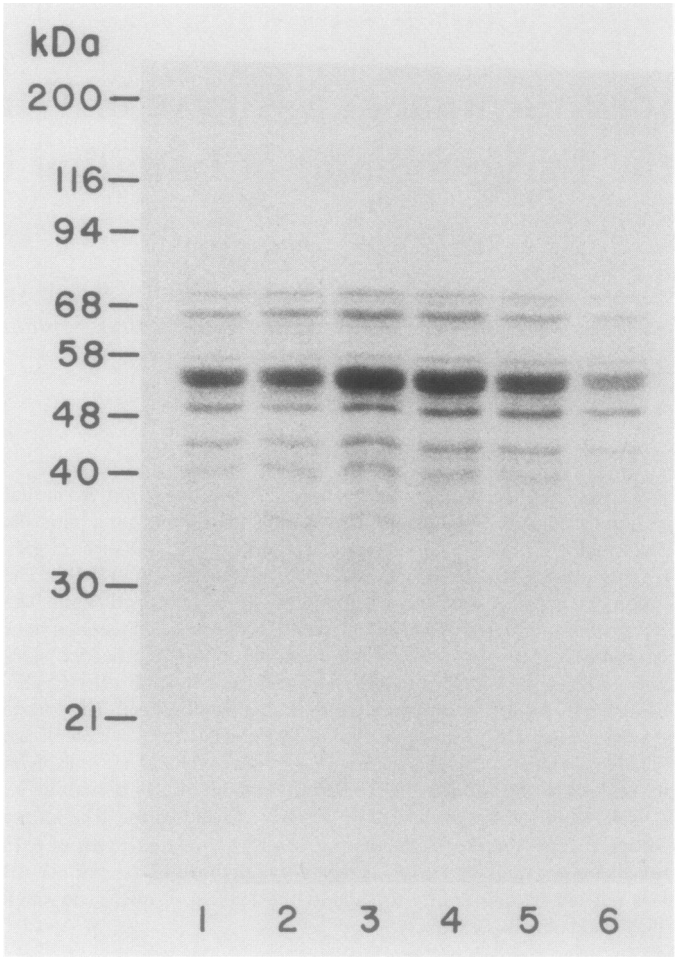

FIG. 2. Analysis of the Sephacryl S-200 II fractions by PAGE in the presence of SDS. Gel electrophoresis was carried by a modification of the procedure of Laemmli (2, 12). The gel contained 12.5% acrylamide and 0.07% bisacrylamide and was stained with Coomassie brilliant blue. Lane 1, 4 μg S-200 I; lanes 2–6, 5 μL of fractions 40, 42, 44, 46, and 48, respectively, from the Sephacryl S-200 II column.

RESULTS AND DISCUSSION

Purification of the Kinase. Preliminary experiments indicated that the 0 to 40% ammonium sulfate fraction obtained from the postribosomal supernatant of wheat germ extracts contains a kinase that phosphorylates eIF-4F. Further work showed that this kinase elutes from DEAE-cellulose in 150 mM KCl (after eIF-4F and before eIF-3 elute from the column (14). The kinase was purified further by fractional precipitation with ammonium sulfate and chromatography on Sephacryl S-200. A summary of the purification of the kinase is given in Table I, and the elution profile of the second Sephacryl S-200 column is shown in Figure 1. The kinase activity elutes from the Sephacryl S-200 slightly in front of ovalbumin (M_r = 45,000). Analysis of the Sephacryl S-200 fractions by SDS-gel electrophoresis (Fig. 2) shows that the active fractions contain several polypeptides. No obvious correlation between an increase in activity and an increase in one or more of the polypeptides is seen and therefore, it is not known which polypeptide(s) is responsible for the kinase activity. The kinase does not bind to casein-Sepharose or to phosphocellulose, and thus far, attempts to further purify the kinase have been unsuccessful.

Table II. *Substrate Specificity of the Kinase*

The reaction mixture described in "Materials and Methods" contained 25 pmol of the initiation factors, elongation factors or ribosomes, 25 μg of casein, phosvitin, or histone IIa and 0.6 μg of the S-200 II kinase preparation.

Substrate	Pi Bound
	pmol
None	<1
Casein	26
Phosvitin	3
Histone IIa	21
eIF-4F	52
eIF-4B	17
eIF-4A	<1
eIF-4G	7
eIF-3	<1
eIF-2	<1
Co-eIF-2βγ	<1
EF-1α	<1
EF-1β	<1
EF-2	<1
40S subunits	<1
60S subunits	3

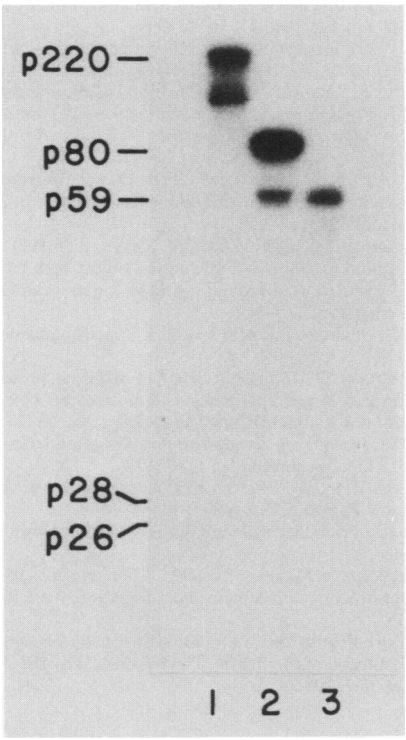

FIG. 3. Analysis of the products of phosphorylation by PAGE in the presence of SDS. eIF-4F, eIF-4B, and eIF-4G were phosphorylated as described in Table II; gel electrophoresis was carried out as described in Figure 2, and the gel was exposed overnight to x-ray film. Lane 1, 3 μg of eIF-4F; lane 2, 2 μg of eIF-4B; and lane 3, 0.6 μg of eIF-4G.

Specificity of the Kinase. The ability of the kinase to phosphorylate other wheat germ initiation factors, wheat germ elongation factors and ribosomes, and several proteins that serve as substrates for other kinases was determined. The results given in Table II show that casein and histone IIA are phosphorylated to a small extent by the kinase; very little phosphorylation of phosvitin is observed. Of the initiation factors tested only eIF-4F, eIF-4B, and, to a lesser extent, eIF-4G are phosphorylated.

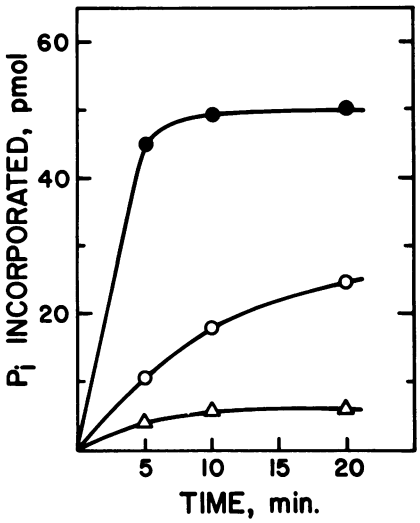

FIG. 4. Rate and extent of phosphorylation of eIF-4F, eIF-4B, and eIF-4G by the kinase. eIF-4F (8 μg, 25 pmol), eIF-4B (2.8 μg, 25 pmol) and eIF-4G (1.5 μg, 25 pmol) were incubated with 0.5 μg of the S-200 II kinase preparation for the times indicated under the conditions described in "Materials and Methods." eIF-4F (●——●); eIF-4B (○——○); eIF-4G (△——△).

Table III. *Effect of Phosphorylation of eIF-4F on its Ability to Support Binding of mRNA to 40S Ribosomal Subunits*

eIF-4F (8 μg) was incubated with 0.6 μg of the S-200 II kinase preparation in the absence of ATP (sham phosphorylated) and in the presence of unlabeled ATP (phosphorylated) under the conditions described in "Material and Methods." The treated and untreated eIF-4F were tested for the ability to support the binding of [^{32}P] satellite tobacco necrosis virus (STNV) RNA (12 pmol) to 40S ribosomal subunits as previously described (8).

eIF-4F Added		[^{32}P]STNV RNA Bound
	μg	*pmol*
None		0.3
Untreated	0.25	0.8
	0.5	1.5
Sham phosphorylated	0.5	1.8
Phosphorylated	0.5	1.5

The elongation factors and the 40S ribosomal subunits are not phosphorylated to a significant extent, and only a small amount of phosphorylation of the 60S ribosomal subunits is observed. The same results were obtained when the proteins listed in Table II were incubated with the kinase and were precipitated with cold 10% trichloroacetic acid and collected on glass fiber filters.

To determine which of the polypeptides of eIF-4F, eIF-4B, and eIF-4G are phosphorylated by the kinase, the reaction mixtures were analyzed by SDS gel electrophoresis followed by autoradiography. The data in Figure 3 show that the 220 kD subunit of eIF-4F and the 80 kD subunit of eIF-4B are phosphorylated by the kinase. These data and other previously published data (1, 13, 15) support the view that the factor which we have designated eIF-4B is probably an isozyme form of eIF-4F. No phosphorylation of the 26 kD subunit of eIF-4F or the 28 kD subunit of eIF-4B is observed. The component in the eIF-4G preparation that is phosphorylated by the kinase is a 59 kD polypeptide which is the size of the active component of eIF-4G (7). The eIF-4B used in the experiment shown in Figure 3 contained a small amount of a 59 kD polypeptide, not found in most eIF-4B preparations, that is also phosphorylated by the kinase. The phosphorylation of a 16 kD component in the 60S

ribosomal subunit preparation can be detected, but only after longer exposure of the polyacrylamide gel (data not shown).

The data in Figure 4 show the rate and extent of phosphorylation of eIF-4F, eIF-4B, and eIF-4G by the kinase. eIF-4F is phosphorylated rapidly by the kinase, reaching close to maximal incorporation within 10 min. At saturation approximately 2 mol of phosphate are incorporated per mol of eIF-4F (M_r = 320,000) (13), all of which is incorporated into the 220 kD subunit. Phosphorylation of eIF-4B (M_r = 110,000) (13) occurs more slowly, taking about 20 min for maximal incorporation to be reached. At saturation approximately 1 mole of phosphate is incorporated per mol of eIF-4B. The amount of phosphate incorporated into eIF-4G after 20 min is about 0.25 mol per mol of eIF-4G and the amount does not increase when the reaction is incubated for longer periods of time. Two possible explanations for this result are: (a) there are two polypeptides in the eIF-4G preparation which have mol wt of approximately 59,000, only one of which can be phosphorylated or (b) there is one polypeptide with a mol wt of 59,000, the majority of which is already phosphorylated.

Effect of Phosphorylation on the Activity of eIF-4F. It was not possible to determine the effect of phosphorylation on the ability of eIF-4F and eIF-4B to support polypeptide synthesis because the factors are dephosphorylated during the course of the reaction. The 40 to 70% ammonium sulfate fraction present in the polymerization system contains a phosphatase(s) that dephosphorylate eIF-4F and eIF-4B. The effect of phosphorylation on the ability of eIF-4F (and eIF-4B) to support the binding of mRNA to 40S ribosomes was measured in a system containing highly purified eIF-2, eIF-3, eIF-4A, and eIF-4G (8). Under these conditions very little dephosphorylation of the factors occurs. The results in Table III show that phosphorylation of eIF-4F does not affect its ability to support the binding of mRNA to 40S ribosomal subunits. Similar results were obtained with eIF-4B (data not shown).

The role of kinases from wheat germ that phosphorylate protein synthesis initiation factors and ribosomal subunits has not been established. Aquino and Tao (3) recently showed that the target protein of a previously described wheat germ kinase (6) inhibits protein synthesis whether or not the target protein is phosphorylated. Further work will be necessary to determine whether phosphorylation/dephosphorylation of the protein synthesis initiation factors plays a role in the regulation of protein synthesis in plant cells.

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